



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12Q 1/68		A1	(11) International Publication Number: WO 00/60116 (43) International Publication Date: 12 October 2000 (12.10.00)
(21) International Application Number: PCT/US00/08465 (22) International Filing Date: 31 March 2000 (31.03.00)		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(30) Priority Data: 60/127,480 2 April 1999 (02.04.99) US 60/169,618 8 December 1999 (08.12.99) US		Published <i>With international search report.</i>	
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(54) Title: HIGH THROUGHPUT AND HIGH SENSITIVITY DETECTION ASSAYS

(57) Abstract

The present invention relates to methods for performing high-throughput biological assays, including immunoassays, nucleic acid detection assays, and related assays. In a preferred embodiment, the target analyte to be detected is a specific mRNA. In another embodiment, the assay monitors the impact of a particular substance or effector on transcription. Alternatively, the target analyte may be a protein, hapten, non-biological chemical, pharmaceutical or other target material. The assay provides high amplification to maximize signal, and utilizes samples which do not require considerable purification.

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TITLE OF THE INVENTION**HIGH THROUGHPUT AND HIGH SENSITIVITY DETECTION ASSAYS****CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims priority to U.S. Provisional Applications, Serial Nos.

- 5 60/127,480, filed April 2, 1999 and 60/169,618, filed December 8, 1999, which are incorporated herein by reference in their entireties.

BACKGROUND OF THE INVENTION**Field of the Invention**

The present invention relates to methods for performing high-throughput

- 10 biological assays, including immunoassays, nucleic acid detection assays, and related assays. In a preferred embodiment, the target analyte to be detected is a specific mRNA, and the assay monitors the impact of a particular substance or effector on transcription. Alternatively, the target analyte may be a protein, hapten, non-biological chemical, pharmaceutical or other target material.

15 **Description of the Related Art**

Typically, in biological assays, a target sample is inspected for the presence and/or amount of a particular analyte. Both immunoassays and nucleic acid detection assays require detection to be specific, that is, a "positive" indication should be given only because of the presence of the target analyte, and not triggered by other factors.

- 20 Simultaneously, the signal must be strong, and easily detected.

Assays for detection of target analytes have high throughput requirements when a wide variety of possible targets must be screened. In the case of nucleic acid detection, many hundreds, if not thousands of individual sequences must be screened to detect the presence of a particular sequence. Developing technology relies heavily on high 5 throughput screening (HTS) not only for detection of a specific target analyte, but also for determining the possible effects of potential drugs, toxins and other effectors on the expression levels of various genes. Many times these assays focus on the level of expression of mRNA in a cell contacted with the effector substance.

When the target analyte is RNA, several methods, including cDNA macro 10 (membrane) and microassays, oligo arrays, and real-time PCR, can be used for RNA detection. However, these methods are extremely expensive and require pure RNA for adequate sensitivity. Furthermore, in oligo arrays, extensive sample manipulation and labeling is required to detect the RNA.

As a result of the limitations on the current procedures, HTS of microtiter well 15 plates or similarly prepared sequences of small chambers that constitute reaction chambers is becoming the method of choice for biological assays. In HTS, hundreds of thousands of combinations of potential activities, samples, probes, and agents are combined and subjected to the same reaction conditions. Each well is then inspected to determine the presence and strength of a particular signal, such as chemiluminescent, 20 colorimetric, fluorescent, or some other visibly detectable signal.

Currently, HTS technology is addressed through two processes. One involves the formation of complex microarrays of literally hundreds of thousands of distinct nucleic acid probes affixed to the surface of an appropriate carrier, such as the silicon dioxide surface of a microchip, a glass slide, or the like. These are exposed to the sample

suspected of containing the target analyte, and then binding events between the probes affixed to the surface and components in the sample inspected are detected. The preparation of the microarrays is time and labor intensive, and adds substantial expense to the technology. In addition, the resulting data are not highly reproducible, in part 5 because mRNA must be purified. This last assay requirement is not compatible with the HTS format.

Another approach to the HTS format involves the use of multiple well microplates. These plates, typically plastic although they may be made from glass as well, have a large number of wells in the plate, typically 96, 384 or greater. The binding 10 reactions necessary to detect the particular sample go on in the well. In conventional assays, the well is coated with a reactant, for example, a DNA probe or an antibody, then the sample is added, with capture occurring if the target analyte is present in the sample. The well must then be washed to remove all unbound material. One of the greatest difficulties encountered in HTS technology is thorough washing of the wells. Material 15 tends to be deposited at the junction of the bottom and the sides of the wells, and thorough washing of the wells, without disrupting the assay materials themselves, is difficult. This technology continues to be plagued, as well, with the difficulty encountered in providing highly specific detection assays with an easily detected, high amplitude signal.

20 Therefore, in view of the aforementioned deficiencies attendant with prior art methods of detecting target analytes, it should be apparent that there still exists a need in the art for a method of detection which is less time and labor intensive, less expensive, and provides for specific identification and ease of detection of a target analyte.

SUMMARY OF THE INVENTION

In accordance with the present invention, it is a principal object of the present invention to provide a method and associated assay system for detecting a target analyte.

It is a further object of the present invention to provide a method and associated assay system for screening substances such as drugs, agents and the like, which have an effect on the level of expression of various genes.

It is a specific object of the invention to provide a method for high throughput detection of the presence or activity of a target analyte, wherein the target analyte is detected by: (a) culturing cells comprising the target analyte at a plurality of positions on a solid surface; (b) lysing the cells to expose the target analyte; (c) contacting the lysed cells with a binding partner such that the binding partner binds to the target analyte to form a complex; (c) detecting formation of the complex; and (d) correlating the presence of the complex with the presence of the target analyte.

It is another specific object of the invention to provide a method for high throughput detection of the presence or activity of target analytes, wherein the target analytes are detected by: (a) spotting multiple probes for the target analytes at a plurality of positions on a solid surface; (b) contacting the probes with cell lysates or non-purified RNA, wherein the cell lysates or non-purified RNA may comprise one or more of the target analytes, such that the probes bind the target analytes to form complexes; (c) detecting formation of the complexes; and (d) correlating the presence and position of each complex detected with the presence of a specific target analyte.

Yet another specific object of the invention is to provide a method of testing the ability of a drug or other entity to modulate the expression of a target analyte which includes:

(a) culturing cells which express the target analyte at a plurality of positions on a solid surface; (b) contacting the cells with the drug under test; (c) lysing the cells to expose the target analyte; (d) contacting the lysed cells with a binding partner such that the binding partner binds to the target analyte to form a complex; (e) detecting formation of the complex; (f) comparing the amount of complex formed in the presence of the drug to the amount of complex formed in the absence of the drug; and (g) correlating a difference in the amount of complex formed in the presence and absence of the drug with the ability of the drug to affect the expression of a target analyte.

Still another specific object of the invention is to provide a method of testing the ability of a drug or other entity to modulate the expression of target analytes which includes :

(a) spotting multiple probes for the target analytes at a plurality of positions on a solid surface to form a mini-array; (b) incubating a sample of cells with the drug under test; (c) lysing the cells to expose the target analytes; contacting the lysed cells with the mini-array formed in (a) such that target analytes present in the lysed cells bind to the probes to form complexes; (d) detecting the presence and position of complexes formed in (c); and

correlating a difference in the amount and/or position of complex formed in the presence and absence of the drug with the ability of the drug to affect the expression of a target analyte.

Other objects and advantages will become apparent to those skilled in the art from a review of the ensuing description which proceeds with reference to the following illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows features of the mRNA detection assay. A long (400-2000bp) single stranded DNA probe which is antisense to specific mRNA is directly attached to a solid support (pin or particle). A detection reagent with high amplification power per 5 single binding event is used, an easy washing step is performed and multiple genes can be read out from the same cell population after signal application.

Figure 2 shows the covalent attachment of long single stranded DNA (ssDNA) probes to solid surfaces, including a microplate, particle and pin.

Figure 3 shows a method of detection with a reagent with enhanced signal 10 amplification power based on an antibody specific to DNA/RNA hybrids. The goal of this detection method is to maximize signal amplification from a single DNA/RNA binding event. A dendromer may be coated with (1) a secreted alkaline phosphatase (SEAP) or selectively mutagenized alkaline phosphatase (AP); and/or (2) antibody(ies).

Figure 4 shows steps involved in an enzyme-linked immunosorbant assay 15 (ELISA)-type assay using a branched antibody/AP complex as a detection reagent.

Figure 5 shows cell-based HTS monitoring of the level of a specific mRNA in reference to a "housekeeping gene" and several other genes.

Figure 6 shows HTS using magnetic pin technology.

Figure 7 shows the membrane/particle approach in a capture-type assay.

20 Figure 8 shows HTS using the membrane/particle approach.

Figure 9 shows a mini-gene array in a 96-well microplate.

Figure 10 shows model oligo detection formats.

Figure 11 shows RNA quantitation in an array format with anti-hybrid detection.

Alternative capture probe formats are shown.

Figure 12 shows model systems for RNA quantitation with anti-hybrid detection - capture probe formats.

Figure 13 shows a Zip-Code array with anti-hybrid detection.

Figure 14 shows a method for performing a high throughput mRNA screening
5 assay using a RIANA biosensor.

Figure 15 shows a method of signal amplification using an RNA/DNA hybrid specific antibody in mRNA detection.

Figure 16 is a graphical comparison of the features of the invention compared with current protocols.

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DETAILED DESCRIPTION

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994)]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M.J. Gait ed. 15 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription And Translation" [B.D. Hames & S.J. Higgins, eds. (1984)]; "Animal Cell Biology" Volumes I-III [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes 5 double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the 10 mRNA).

The term "oligonucleotide," as used herein in referring to the probe of the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

15 The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA 20 polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use of the method. For example, for diagnostic applications, depending on the complexity of

the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be 5 sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, 10 provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

Two DNA sequences are "substantially homologous" when at least about 75% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the 15 nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis et 20 al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule. (See Weintraub, 1990; Marcus-Sekura, 1988.) In the cell, they hybridize to that mRNA, forming a double stranded molecule. The cell does not translate an mRNA in this double-stranded form. Therefore, antisense

nucleic acids interfere with the expression of mRNA into protein. Antisense methods have been used to inhibit the expression of many genes *in vitro* (Marcus-Sekura, 1988; Hambor et al., 1988).

Two amino acid sequences are "substantially homologous" when at least about 5 70% of the amino acid residues (preferably at least about 80%, and most preferably at least about 90 or 95%) are identical, or represent conservative substitutions.

An "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses polyclonal, monoclonal, and chimeric antibodies, the last mentioned described in further detail in U.S. Patent

10 Nos. 4,816,397 and 4,816,567.

An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

The phrase "antibody molecule" in its various grammatical forms as used herein 15 contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule.

Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the paratope, including those portions known in the art as Fab, 20 Fab', F(ab')₂ and F(v), which portions are preferred for use in the methods described herein.

Fab and F(ab')₂ portions of antibody molecules are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See for example, U.S. Patent No. 4,342,566 to

Theofilopolous et al. Fab' antibody molecule portions are also well-known and are produced from F(ab')₂ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody 5 containing intact antibody molecules is preferred herein.

The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may 10 therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) monoclonal antibody.

The term "standard hybridization conditions" refers to salt and temperature conditions substantially equivalent to 5 x SSC and 65°C for both hybridization and 15 wash. However, one skilled in the art will appreciate that such "standard hybridization conditions" are dependent on particular conditions including the concentration of sodium and magnesium in the buffer, nucleotide sequence length and concentration, percent mismatch, percent formamide, and the like. Also important in the determination of "standard hybridization conditions" is whether the two sequences hybridizing are RNA- 20 RNA, DNA-DNA or RNA-DNA. Such standard hybridization conditions are easily determined by one skilled in the art according to well known formulae, wherein hybridization is typically 10-20°C below the predicted or determined T_m with washes of higher stringency, if desired.

The degree of similarity between the nucleic acid sequences of two polynucleotides may be measured by determining whether the two polynucleotide sequences can hybridize to each other under a given set of conditions. Accordingly, one aspect of the invention is directed to polynucleotide molecules capable of selectively hybridizing to a target under stringent hybridization conditions, and the use thereof in diagnostic assays.

5 These hybridization conditions may be varied so that the hybridization interaction between the two polynucleotide sequences occurs at a certain number of degrees centigrade below the melting temperature (Tm) of the duplex polynucleotide molecule 10 used as the hybridization probe.

“Tm” is defined as the temperature at which half the duplex molecules have melted or dissociated into their constituent single strands. The Tm for a given double-stranded polynucleotide may be determined empirically or by reference to well known formulas that take into account hybridization conditions that influence Tm. For DNA - 15 DNA hybridization probes longer than 50 nucleotides, $Tm = 81.5^{\circ}\text{C} + 16.6 \log M$ (M represents molar divalent cation concentration) + 41 (mole fraction G + C) -500/L - 0.62 (% formamide), as described in Berger and Kimmel (1987) *Guide to Molecular Cloning Techniques, Methods in Enzymology*, Vol. 152, Academic Press, San Diego CA.

20 The “stringency” of a hybridization may be defined as degrees centigrade below the Tm of the probe nucleotide sequence. The degree of stringency of hybridization is said to decrease as hybridization takes place at a temperature increasingly below the Tm of the hybridization probe. Maximum stringency typically occurs at about $Tm - 5^{\circ}\text{C}$ (at a temperature 5°C below the Tm of the hybridization probe). “High stringency” hybridization is said to take place at a temperature of about 5°C to 10°C below Tm.

“Intermediate stringency” hybridization is said to take place at a temperature of about 10°C to 20°C below Tm. “Low stringency” or maximum hybridization is said to take place at a temperature of about 20°C to 25°C below Tm.

Nucleic acid hybridization probes for the detection of a target mRNA should 5 preferably hybridize to at least 50% of the nucleotides from the sequence of a given nucleotide sequence. Hybridization probes may be labeled by a variety of reporter groups, including, but not limited to, radionuclides such as ^{32}P or ^{35}S , or enzymatic labels such as horseradish peroxidase or alkaline phosphatase coupled to the probe via avidin/biotin coupling systems.

10 Probes for hybridization may be synthesized by both enzymatic and *in vitro* techniques. Short hybridization probes are preferably synthesized by *in vitro* methodology on commercially available DNA synthesizers such as the machines sold by Applied Biosystems. If longer sequences are of interest, oligonucleotides produced by *in vitro* synthesis may be readily spliced together using generally known ligation 15 techniques. The oligonucleotide probes will generally comprise between about 10 nucleotides and several thousand nucleotides. For detection of a specific mRNA, an optimal probe is about 100-10,000 nucleotides, preferably 200-5000 nucleotides, and preferably between about 400-2000 nucleotides.

Other means of producing target analyte-specific hybridization probes include the 20 cloning of nucleic acid sequences of the target analyte into vectors for the production of RNA probes. Such vectors are known in the art, commercially available, and may be used to synthesize RNA probes *in vitro*. The vector of interest is labeled by addition of the appropriate RNA polymerases, such as T7 or SP6 RNA polymerase, and the

appropriate labeled nucleotides, including, but not limited to, fluorescent or radioactive nucleotides.

In its primary aspect, the present invention concerns the identification of a target analyte. Advantages of the present invention include high sensitivity and the ability to use non-purified samples as a source for identifying the target analyte. The assay may utilize one of two general formats: (1) wherein cells are cultured at a plurality of positions on a solid surface and a probe for the target analyte contacted therewith; or (2) wherein probes for the target analyte are spotted at a plurality of positions to form a "micro-array", and lysed cells or non-purified fractions or components of cells (e.g., non-purified RNA) are contacted therewith. Both formats preferably utilize probes which are highly specific for the specific target analyte, and utilize amplification procedures which maximize the signal obtained when the probe (also referred to as a "binding partner") binds to the target analyte.

As described in detail above, antibody(ies) to the target analyte, in particular to an RNA/DNA hybrid, can be produced and isolated by standard methods including the well known hybridoma techniques. For convenience, the antibody(ies) to the target analyte or DNA/RNA hybrid will be referred to herein as Ab_1 , and antibody(ies) raised in another species as Ab_2 .

The presence of a target analyte, including a specific mRNA, in cells can be ascertained by the usual immunological procedures applicable to such determinations. A number of useful procedures are known. Three such procedures which are especially useful utilize either the target analyte labeled with a detectable label, a DNA which binds a specific mRNA labeled with a detectable label, antibody Ab_1 labeled with a detectable label, or antibody Ab_2 labeled with a detectable label. The procedures may be

summarized by the following equations wherein the asterisk indicates that the particle is labeled, and "TA" stands for the target analyte:

- A. $TA^* + Ab_1 = TA^*Ab_1$,
- B. $TA + Ab_1^* = TAAb_1^*$
- 5 C. $TA + Ab_1 + Ab_2^* = TAAb_1Ab_2^*$

The procedures and their application are all familiar to those skilled in the art and accordingly may be utilized within the scope of the present invention. The "competitive" procedure, Procedure A, is described in U.S. Patent Nos. 3,654,090 and 3,850,752.

Procedure C, the "sandwich" procedure, is described in U.S. Patent Nos. RE 31,006 and

10 4,016,043. Still other procedures are known such as the "double antibody," or "DASP" procedure.

In each instance, the target analyte forms complexes with one or more antibody(ies) or binding partners (in the case of an mRNA target, the binding partner is a DNA probe) and one member of the complex is labeled with a detectable label. The fact 15 that a complex has formed and, if desired, the amount thereof, can be determined by known methods applicable to the detection of labels.

It will be seen from the above, that a characteristic property of Ab_2 is that it will react with Ab_1 . This is because Ab_1 raised in one mammalian species has been used in another species as an antigen to raise the antibody Ab_2 . For example, Ab_2 may be raised 20 in goats using rabbit antibodies as antigens. Ab_2 therefore would be anti-rabbit antibody raised in goats. For purposes of this description and claims, Ab_1 will be referred to as a primary or anti-TA antibody, and Ab_2 will be referred to as a secondary or anti- Ab_1 antibody.

The labels most commonly employed for these studies are radioactive elements, enzymes, chemicals which fluoresce when exposed to ultraviolet light, and others.

A number of fluorescent materials are known and can be utilized as labels. These include, for example, fluorescein, rhodamine, auramine, Texas Red, AMCA blue and 5 Lucifer Yellow. A particular detecting material is anti-rabbit antibody prepared in goats and conjugated with fluorescein through an isothiocyanate.

The target analyte or its binding partner(s) can also be labeled with a radioactive element or with an enzyme. The radioactive label can be detected by any of the currently available counting procedures. The preferred isotope may be selected from ^3H , ^{14}C , ^{32}P , 10 ^{35}S , ^{36}Cl , ^{51}Cr , ^{57}Co , ^{58}Co , ^{59}Fe , ^{90}Y , ^{125}I , ^{131}I , and ^{186}Re .

Enzyme labels are likewise useful, and can be detected by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques. The enzyme is conjugated to the selected particle by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Many enzymes which can be used in these procedures are known and can be utilized. The preferred are peroxidase, β -glucuronidase, β -D-glucosidase, β -D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase.

U.S. Patent Nos. 3,654,090; 3,850,752; and 4,016,043 are referred to by way of example for their disclosure of alternate labeling material and methods. It is also particularly preferred to use an enzyme which has been modified to reduce non-specific binding. 20 This can be done, for example, by utilizing a secreted form of the enzyme, or by site-directed mutagenesis of the enzyme.

In a further embodiment of this invention, commercial test kits suitable for use by a medical specialist may be prepared to determine the presence or absence of a

predetermined target analyte in suspected target cells. In accordance with the testing techniques discussed above, one class of such kits will contain at least the labeled binding partner to the target analyte, for instance a probe or an antibody specific thereto, and directions, of course, depending upon the method selected, e.g., "competitive," 5 "sandwich," "DASP" and the like. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

Accordingly, a test kit may be prepared for the demonstration of the presence or capability of cells for the presence of a target analyte, comprising:

- (a) a predetermined amount of at least one labeled immunochemically reactive 10 component obtained by the direct or indirect attachment of the target analyte or a specific binding partner thereto, to a detectable label;
- (b) other reagents; and
- (c) directions for use of said kit.

More specifically, the diagnostic test kit may comprise:

- (a) a known amount of the target analyte as described above (or a binding partner) 15 generally bound to a solid phase to form an immunosorbent, or in the alternative, bound to a suitable tag, or plural such end products, etc. (or their binding partners) one of each;
- (b) if necessary, other reagents; and
- (c) directions for use of said test kit.

20 In a further variation, the test kit may be prepared and used for the purposes stated above, which operates according to a predetermined protocol (e.g. "competitive," "sandwich," "double antibody," etc.), and comprises:

- (a) a labeled component which has been obtained by coupling the target analyte to a detectable label;

(b) one or more additional immunochemical reagents of which at least one reagent is a ligand or an immobilized ligand, which ligand is selected from the group consisting of:

- (i) a ligand capable of binding with the labeled component (a);
 - (ii) a ligand capable of binding with a binding partner of the labeled component (a);
 - (iii) a ligand capable of binding with at least one of the component(s) to be determined; and
 - (iv) a ligand capable of binding with at least one of the binding partners of at least one of the component(s) to be determined; and
- 10 (c) directions for the performance of a protocol for the detection and/or determination of one or more components of an immunochemical reaction between the target analyte and a specific binding partner thereto.

In accordance with the above, an assay system for screening potential drugs effective to modulate the level of expression of the target analyte may be prepared. The 15 prospective drug may be introduced into a cell culture, and the culture thereafter examined to observe any changes in the expression of the target analyte.

Initially, the invention utilizes a probe (e.g., a denatured PCR product, a single-stranded DNA, or a hybrid capture II probe) or in an alternative embodiment, antibody complexes, which are specific for a target analyte sequence.

20 The probe may be covalently bound directly to a solid support, which provides for convenience in handling. Preferably, the solid support is a 96 well microtiter plate or nylon membrane. Alternatively, the probe can be attached to a "pin" or columnar device located in the well of a microtiter plate. Because the probe is specific, it is necessary to maximize the signal detected as a result of the binding.

In one embodiment, the probe may be biotinylated. The biotin can then be detected with a labeled avidin molecule, or alternatively, an unlabeled avidin may be detected with a labeled avidin antibody.

In the embodiment wherein mRNA is to be detected, cells may be cultured, or 5 non-purified RNA may be spotted onto a solid support, and a DNA probe for the target mRNA is introduced. Alternatively, the DNA probe may be attached to the solid surface, and lysed cells or non-purified RNA introduced. After binding of the target mRNA to the DNA probe, a labeled antibody to RNA/DNA complexes may be added.

The "label" or "reporter molecule" may be an enzyme for chemiluminescent 10 detection of a fluorescent label for direct detection. Alternatively, the label may be a luminescent molecule, such as a dioxetane, which may be activated by enzymes. In a preferred commercial embodiment, the enzyme is alkaline phosphatase, particularly secreted alkaline phosphatase (SEAP), and the reporter substrate on which the enzyme acts is a dioxetane such as AMPPD®. Other dioxetanes, such as CSPD® and CDP- 15 *Star*®, all available from Tropix, Inc. of Bedford, Massachusetts may similarly be used.

In these embodiments, when there is a selective capture after washing, the dioxetane substrate is added, and caused to decompose by the presence of the enzyme. On decomposition, the dioxetane reporter molecule releases light. Other luminescent molecule systems are well known and can be used instead.

20 The image signal from the label or reporter molecule can be read with a sensitive high resolution CCD camera, luminometer, or scanning fluorescent reader.

To amplify the enzyme in order to give an improved signal-to-noise (S/N) ratio, multiple enzyme molecules may be associated with a single binding probe. In one embodiment, dendromers, available from Polyprobe, Inc., are coated with multiple

enzyme molecules and at least one antibody. The ideal ratio of enzyme to antibody is determined based on the nature of the assay. In an alternative methodology, a DNA probe is attached to a column with a restriction site built-in such that the DNA can be subsequently released from the column. The DNA linker is biotinylated. The column is 5 then loaded with avidin or streptavidin to saturate the biotin present on the DNA linker. Each streptavidin molecule binds to four biotin molecules, and accordingly, bis-biotin (available from Pierce) is loaded on the column, and bound to the streptavidin. Streptavidin is then added, again, in a repeated sequence, to maximize the number of 10 streptavidin units available. Ultimately, biotinylated enzyme and the antibody specific for the nucleic acid hybrid target are added, the biotin binding to unbound streptavidin secured in the matrix.

When sufficient enzyme/antibody/streptavidin complex has been built-up, the reagent is released from the column by addition of the restriction enzyme. Unbound material remains trapped in the column. These processes result in a highly specific 15 reagent with a high signal amplification.

Different assay methodologies may be employed. In a first embodiment, HTS screening is employed, using commercially available microplates, but the reagent is attached not to the microplate, but to pins which may be lowered into and raised out of the sample or solution added to the microplate. Thus, each well may be exposed to a 20 single or plurality of pins (e.g., an eight-pin set) with every pin coated with a different ss DNA probe, prepared as described above. The target sample, for example cells, are placed in the well and lysed to release the suspected target analyte. The target mRNA, if present, will hybridize to the proper probe on the pins. The pins are removed from the first well, and a second well is provided which contains the antibody/enzyme detection

reagent, the two being incubated together. The pins may be removed again, and washed with an appropriate buffer. Finally, the pins are exposed to wells provided with the reporter substrate, such as a dioxetane, and read conveniently by automated machinery such as a CCD or luminometer. In this way, no wells need be washed, multiple genes 5 can be exposed to the same cell lysis product, and a highly specific and sensitive assay is provided. The pins may either be plastic or coated glass, for each of attachment, or the particles coated by the ss DNA probe may be magnetic, and magnetic pins may be used. One advantage of the latter is that the pins may be cleaned and reused. Exemplary pins, used for other purposes, are available from Nalge Nunc International, as replicators, 10 designed for the replication of DNA libraries. Similar apparatus is available from V & P Scientific. Alternate devices may be prepared as well.

An alternate assay relies on membrane capture of bound particles. In this assay, particles coated with the probe, such as the ss DNA probe discussed above, are provided. The particles are provided with nucleic acid that will hybridize to specific mRNA. These 15 particle bound probes are allocated into each well of a multiple well microplate provided with a membrane bottom. Such microplates are available from Nalge Nunc International, generally marketed under the name "Silent Screen". These microplates were more thoroughly discussed in the poster presentation by Nalge Nunc International at SBS September 20-24, 1998 and Drug Discovery, August 10-13, 1998. 20 Polyfiltronics/Whatman and Millipore also supply these microplates.

Target cells are grown on the membrane bottom of the microplate wells, in the presence of the effector desired, and subsequently lysed. Hybridization conditions are maintained, to allow hybridization to occur between the probe bound to the particle, and any complementary mRNA present in the cell lysis mixture. Following hybridization,

the mixture is contacted with the enzyme/antibody conjugate, and again incubated, to allow the antibody present to bind to any nucleic acid hybrid for which it is specific present in the mixture. Thereafter, the micro well is washed. Where the membrane pore size is selected to be slightly less than the particle size, washing under vacuum filtration 5 will remove unbound reagent. The substrate (the dioxetane or other luminescent reporter) is added to the micro plate wells, which now contain only the specifically bound particles, and a strong signal is released. Again, the problem of washing micro plate wells is avoided. In an alternative process, the cell lysis preparation may be done outside the micro plate well, and then added thereto.

10 The above-identified arrays provide several advantages over conventional arrays and other methodologies for the detection of nucleic acids. For instance, in the present invention, a sample containing non-purified RNA or cell lysates can be used. Additionally, the target mRNA is detected with high sensitivity. Further, the proposed device has the advantage of measuring gene expression from multiple genes for a large 15 number of samples in a high throughput pharmaceutical screening format.

In another aspect of the present invention, multiple probes for specific mRNAs are spotted in a mini-array in the bottom of a microplate. The plate bottom surface can be nylon, PVDF, or other material, with either a flow-through or a solid bottom design.

20 The array has applications for gene expression analysis, including, for example, low density macroarrays, high density cDNA arrays, high density oligo arrays and novel HTS formats. The array can also be applied to toxicology or pathway determination by analyzing gene expression. The array can also be used for DNA sequencing (sequencing by hybridization or SBH) for use in genomics research or diagnostic sequencing. It can also be used for genetic mapping, including assessment of single nucleotide

polymorphisms (SNPs) and disease linkage. The array is also useful for pharmacogenetics (SNP and other). The arrays may also be comprised of peptide/protein or cells (SCIMS, Cellomics).

The array may be used in combination with a "stripped down" Northstar system.

5 12 x 8 cm membrane arrays can be made with 96-5,000 features. Detection may be performed using chemiluminescence.

Other aspects of the arrays may involve the use of porous silicon, polymer capture, matrix localization and microcompartmentalization. Mini-gene expression arrays are useful for increased information content for high-throughput screening.

10 For spotting the array, a pen quill instrument designed for glass slide spotting may be used with a spot volume on the membrane of 1 - 5 nL (Foster City, S&T).

Membranes which may be used include, but are not limited to PVDF .45 um (MSI), UltraBind \AA polyether sulfone (preactivated to react with primary amines) (Pall), nylon 6,6 membranes including Biodyne A .45 um, Biodyne Plus .45 um, XuXu neutral 15 unsupported nylon .45 um*, NY0011 .2 um unsupported Biodyne B/.8 um supported Biodyne B*, Biodyne A composite .04 um*, Biodyne A .1 um*, Biodyne B, H support .45 um*, Immunodyne \AA ABC .45 um (preactivated to react with primary amines), Mylar backed Biodyne A .04 um lot N, Mylar backed Biodyne A .04 um lot O, and Aquarius experimental membrane (Pall).

20 The oligonucleotide attachment may be performed by means of UV X-link or plasma gas derivatization (ammonia, oxygen or hydrogen and ammonia) covalent attachment with DITC and amine labeled oligos.

The goals of using an array for HTS include measuring an expression level of 10-100 genes in a high throughput pharmaceutical screening format, the utilization of non-

purified RNA or cell lysates, and achieving adequate sensitivity for the target mRNA of interest. Array panels may be developed which represent a pharmaceutically relevant groups of genes (i.e., human cancer, human toxicology, viral infection, apoptosis.)

The limitations of current products are that they are not for HTS, they are
5 extremely expensive, they require enzymatic labeling of the target and ug quantities of pure RNA for adequate sensitivity. Known oligo arrays have these limitations and also require extensive sample manipulation and labeling. Real-time PCR requires pure RNA and detection is not high throughput.

The following examples are presented in order to more fully illustrate the
10 preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLES

Example 1

mRNA detection assay

15 The assay is designed to be sensitive enough to detect a single copy of a specific mRNA/cell. Maximum signal amplification is based on an antibody against a specific DNA/RNA hybrid. Detection monitors changes in the level of a specific message referred to as a "housekeeping gene" as well as several other genes. The assay is performed in HTS format.

20 Key features of the assay (Figure 1) are that a long (400-2000bp) single stranded DNA probe in antisense to specific mRNA is used. The probe is attached directly to a solid support. A detection reagent with high amplification power per single binding

event is used. An easy washing step is performed and one can obtain a multiple gene read out from the same cell population after signal application.

A long ssDNA probe (close to the full size of the mRNA target) is covalently attached to the surface (Figure 2) in order to obtain a) effective capture of the 5 mRNA/DNA hybrid on the surface by covalent binding (no signal loss during washing); (b) protection of the mRNA from degradation in the cell lysate by hybridization to the long probe; and (c) simplified preparation of evenly coated surfaces. A homogeneous reagent can be obtained by using probe coated particles.

Methods of producing the probe include run-off PCR using a 5' activated primer 10 or a viral M13 approach.

The detection reagent has enhanced signal amplification power and is based on a DNA/RNA hybrid specific antibody, so as to maximize signal amplification from a single DNA/RNA binding event. The amplification enzyme (alkaline phosphatase) is modified in order to decrease nonspecific binding by either (1) using SEAP (secreted 15 alkaline phosphatase - lacking membrane region) or (2) selective mutagenesis of alkaline phosphatase.

A branched AP/antibody coated dendromer (Polyprobe, Inc.) (Figure 3) is used as the detection reagent for an ELISA-type assay. Preferably, a DNA/RNA hybrid-specific antibody with enhanced signal amplification power is used (Figure 4).

20 **Example 2**

Synthesis of branched complex

(1) A biotinylated double stranded DNA linker with two restriction enzyme sites was attached to a column. (2) The column was loaded with streptavidin in excess to

saturate all biotin. (3) The unbound streptavidin was washed out. (4) The column was loaded with bis-biotin (PIERCE) and incubated. (5) Unbound bis-biotin was washed out. (6) The column was loaded with streptavidin in excess to saturate all free biotin and incubated. (7) Unbound streptavidin was washed out. (8) Steps 4-7 were repeated 5 several times to reach a designated number of “layers” in the matrix. (9) The column was loaded with biotinylated alkaline phosphatase and RNA/DNA hybrid-specific antibody in the desired proportion and incubated. (10) Unbound proteins were washed out. (11) The column was loaded with restriction enzyme in order to free-up formed matrix and incubated. (12) Formed matrix was washed out. (13) Nonspecifically bound 10 streptavidin, bis-biotin, alkaline phosphatase, and antibody was retained in column.

Formed matrix was used as the detection reagent in an mRNA detection assay. The matrix synthesized with corresponding antibody can also be used in any type of ELISA assay or DNA/RNA detection assay.

Example 3

15 Cell based HTS monitoring level of specific mRNA in reference to housekeeping gene and several (6) other genes

A. HTS using pin technology (Figure 5)

1. Coating/blocking.

A microplate lid with pins oriented down was used to create an active surface on 20 the pins for hybridization with a specific mRNA. The active surface was created on pins by incubation in 384 well microplate with a 5' end-activated single stranded DNA. Each pin was incubated (coated) in a separate well containing DNA in antisense to the specific

mRNA. The pins were washed and incubated in a pool with an appropriate blocking buffer. 384 pins were grouped by 8 (4) neighbors pins. One pin in each group was coated with DNA corresponding to the mRNA of the gene of interest. The remainder of the pins were coated with DNA corresponding to the housekeeping gene and mRNA from the reference genes.

5 2. Hybridization.

Cells were grown in a 48 (96) well microplate, starved, induced, incubated with the corresponding compound from the library and lysed. Hybridization buffer was added to the wells. A group of 8 (4) pins was applied in every single well containing cell lysate 10 and incubated.

3. Detection reagent binding.

Detection reagent was added to each well and incubated.

4. Washing.

Pins were placed in a large pool with circulating washing buffer for effectively 15 washing away nonspecifically bound detection reagent.

5. Detection.

Pins were placed in the 384 well microplate with a substrate for alkaline phosphatase. After 30 minutes, the pins were removed and the plate was read by a luminometer.

20 B. HTS using magnetic pin technology (Figure 6)

The method is performed as in (A) above, except that pins are covered by magnetic particles coated with the ssDNA probes.

Example 4

Membrane/particle approach in capture type assay (Figure 7)

Particles are prepared in bulk with a reagent which changes as a result of the reaction and can therefore be monitored. Single stranded DNA in antisense to a specific mRNA are attached to the particles and an aliquot is placed into each well of a membrane bottom microplate (96 well, 384 well, etc.)

Cells are grown on the membrane and induced. A compound from the library is added, and the cells lysed. Hybridization is conducted at 65°C for 2 hours. The AP/antibody conjugate is added and incubated. The wells are then washed 3 times with EW buffer at 53°C, and then washed 2 times with HCII buffer. AP substrate is then added and incubated. The plates are then read.

Example 5

HTS using membrane/particles approach (Figure 8)

Cells can be grown in a well of a 96 well culture microplate, induced, lysed, and transferred into a membrane bottom microplate, or grown directly on a membrane of a membrane bottom microplate, induced and lysed. A sampling from a single well is placed into 8 wells of a 384 well filter plate. Each well is loaded with particles coated with a specific probe. A hybridization is performed, a detection reagent is added and incubated, the wells are washed and then incubated with a substrate, followed by reading on a luminometer.

Example 6

Membrane/particles approach in HTS

One of the major limiting steps in miniaturization of capture based assays in HTS format is washing away nonspecifically bound detection reagent. The surface of particles 5 was used as a capturing surface instead of the walls and bottom of a microplate well.

The surface of particles was coated with reagent in which the modification reaction takes place. The surface was blocked. Treated particles were added to the reaction mix and incubated directly in membrane-bottomed microplate wells (96 well or 384 well). Detection reagent was added to the reaction mix and incubated. The 10 incubation with detection reagent allowed antibody to bind to modified reagent. Washing under vacuum filtration effectively removed nonspecifically bound detection reagent. Alkaline phosphatase substrate was added to microplate wells. After incubation, the microplate was read in a luminometer.

Particles with an enhanced surface were used. This can be considered an 15 advantage compared to the internal surface of a microplate well. The role of the filter membrane was limited to holding particles during washing. Therefore, membrane pore size was chosen to be slightly less than particle size. Nonspecific binding of detection reagent to the membrane was minimized, and background due to interaction of the alkaline phosphatase substrate with the membrane was lowered.

20 **Example 7**

In the Xpress-Screen protocol, particles were coated with streptavidin, followed by attachment of biotinylated DNA/mRNA hybrid. A detection reagent, an antibody

specific for DNA/RNA hybrids, conjugated to alkaline phosphatase, was used to detect formed complex.

Example 8

Particles were coated with a peptide containing a phosphorylation site specific for 5 a kinase of interest. A detection reagent based on an antibody directed to that phosphorylated site was used to detect the results of the reaction.

This approach can be applied to any ELISA or enzymatic incorporation or extension type assay. In general the approach can be applied to any assay where the reaction product is fixed to the surface, which should be spatially separated from the 10 reagent used in intermediate steps.

Polyfiltronic Plates ppl.25um 5ul Seradene

	1	2	3	4	5	6	7	8
A	1751	1047	1288	146	16	17		
B	1940	1497	1573	155	18	20		
C	15855	15022	13812	414	38	15		
D	170317	146279	146262	4412	19	16		
E	130907	143613	133694	6081	17	20		
F	1122804	1034084	1070899	460	22	42		
G	2512786	2301960	2426350	1122	33	20		
H	2818058	2878221	2945755	1575	78	33		
	s/n		stdev		%CV			
	1516	0	319.5484		21.07839			
	14896.33 10x3	9.826077	1027.281		6.896201			
	145178.7 10x4	95.76429	13945.25		9.605576			
	1075929 10x5	709.7157	44573.37		4.14278			
	2413699 10x6	1592.15	105980.9		4.390807			
	2880678 10x7	1900.183	63883.95		2.217671			

Example 9**Mini-Gene Array in 96 Well Microplate - High-Throughput Array Detection (Figure 9)**

Multiple probes for specific mRNAs are spotted in a mini-array in the bottom of a microplate. An RNA sample is added and the assay is performed with standard 96 well microplate liquid handling. The plate bottom surface can be nylon, PVDF, or other material, with either flow-through or a solid bottom design. The signal can be imaged with a sensitive high resolution CCD camera.

A high throughput mRNA screening assay may be performed using Xpress-Screen™ (Tropix Inc.) with applications for the RIANA module (Figure 14). The assay can include the use of a biotinylated DNA capture probe in combination with CDP-*Star*®, or may be a modified assay using the RIANA-biosensor as the detection device: (1) mRNA and fluorescently labeled capture DNA is hybridized to form a DNA-RNA hybrid; (2) the capture DNA-RNA hybrid is decoded onto the biosensor surface using the Zip-Code; and (3) detection is performed using fluorescently labeled antibodies which are highly specific for RNA-DNA hybrids. Through the use of multiplexing, 126 mRNA are detectable at once.

Example 10

A double-stranded, covalently closed M13mp18 phage vector (New England BioLabs) was used for subcloning a 100 bp DNA corresponding to specific target mRNA. Phage is propagated in *E. coli* K12 JM101. The replicative form of DNA was isolated from infected cells and purified by gel filtration and column chromatography.

5 RNA was synthesized *in vitro* from the T7 promoter using most of the 9000 bp of the M13mp18 phage as a template excluding the 100 bp target specific region. Single-stranded phage DNA was hybridized to the synthetic RNA. The RNA/phage DNA hybrid was mixed with cell lysate, two 100 bp single stranded biotinylated capture probes 10 and incubated 3 hours for hybridization of the DNA probes and mRNA target. The mixture was then applied to a single well of a streptavidin coated microplate for overnight capture.

Alkaline phosphatase conjugated antibody against the DNA/RNA hybrid was added. After a 1 hour incubation and washing of unbound antibody and probes, alkaline 15 phosphatase substrate was added. After a 30 minute incubation, chemiluminescent signal was measured.

While the invention has been described and illustrated herein by references to various specific material, procedures and examples, it is understood that the invention is not restricted to the particular material, combinations of material, and procedures 20 selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art.

WHAT IS CLAIMED IS:

1. A method for high throughput detection of the presence or activity of a target analyte, wherein said target analyte is detected by:

- 5 A. culturing cells comprising the target analyte at a plurality of positions on a solid surface;
- B. lysing the cells to expose the target analyte;
- C. contacting the lysed cells with a binding partner such that the binding partner binds to the target analyte to form a complex;
- 10 D. detecting formation of the complex; and
- E. correlating the presence of the complex with the presence of the target analyte.

2. The method of Claim 1, wherein the solid surface is a membrane or microplate.

15

3. The method of Claim 1, wherein the target analyte is a specific mRNA.

4. The method of Claim 3, wherein the binding partner is DNA.

20 5. The method of Claim 1, wherein the binding partner is attached to a pin.

6. The method of Claim 1, wherein the binding partner is attached to a particle.

7. The method of Claim 4, further comprising contacting the complex with an antibody to RNA/DNA hybrids.
8. The method of Claim 7, wherein the antibody is conjugated to enzyme which produces a measurable signal.
9. The method of Claim 8, wherein the enzyme is alkaline phosphatase.
10. The method of Claim 9, wherein the alkaline phosphatase is modified to reduce non-specific binding.
11. A method for high throughput detection of the presence or activity of target analytes, wherein said target analytes are detected by:
 - A. spotting multiple probes for the target analytes at a plurality of positions on a solid surface;
 - B. contacting the probes with cell lysates or non-purified RNA, wherein the cell lysates or non-purified RNA may comprise one or more of the target analytes, such that the probes bind the target analytes to form complexes;
 - C. detecting formation of the complexes; and
 - D. correlating the presence and position of each complex detected with the presence of a specific target analyte.
12. The method of Claim 11, wherein the solid surface is a membrane or microplate.

13. The method of Claim 11, wherein the target analyte is a specific mRNA.

14. The method of Claim 13, wherein the probe is DNA.

5

15. The method of Claim 11, wherein the probe is labeled with a detectable label.

16. The method of Claim 11, further comprising contacting the complex with an antibody to RNA/DNA hybrids.

10

17. The method of Claim 16, wherein the antibody is conjugated to enzyme which produces a measurable signal.

18. The method of Claim 17, wherein the enzyme is alkaline phosphatase.

15

19. A method of testing the ability of a drug or other entity to modulate the expression of a target analyte which comprises:

- A. culturing cells which express the target analyte at a plurality of positions on a solid surface;
- B. contacting the cells with the drug under test;
- C. lysing the cells to expose the target analyte;
- D. contacting the lysed cells with a binding partner such that the binding partner binds to the target analyte to form a complex;
- E. detecting formation of the complex;

- F. comparing the amount of complex formed in the presence of the drug to the amount of complex formed in the absence of the drug; and
- G. correlating a difference in the amount of complex formed in the presence and absence of the drug with the ability of the drug to affect the expression of a target analyte.

5

- 20. A method of testing the ability of a drug or other entity to modulate the expression of target analytes which comprises:
 - A. spotting multiple probes for the target analytes at a plurality of 10 positions on a solid surface to form a mini-array;
 - B. incubating a sample of cells with the drug under test;
 - C. lysing the cells to expose the target analytes;
 - D. contacting the lysed cells with the mini-array formed in (A) such that 15 target analytes present in the lysed cells bind to the probes to form complexes;
 - E. detecting the presence and position of complexes formed in (D);
 - F. correlating a difference in the amount and/or position of complex formed in the presence and absence of the drug with the ability of the drug to affect the expression of a target analyte.

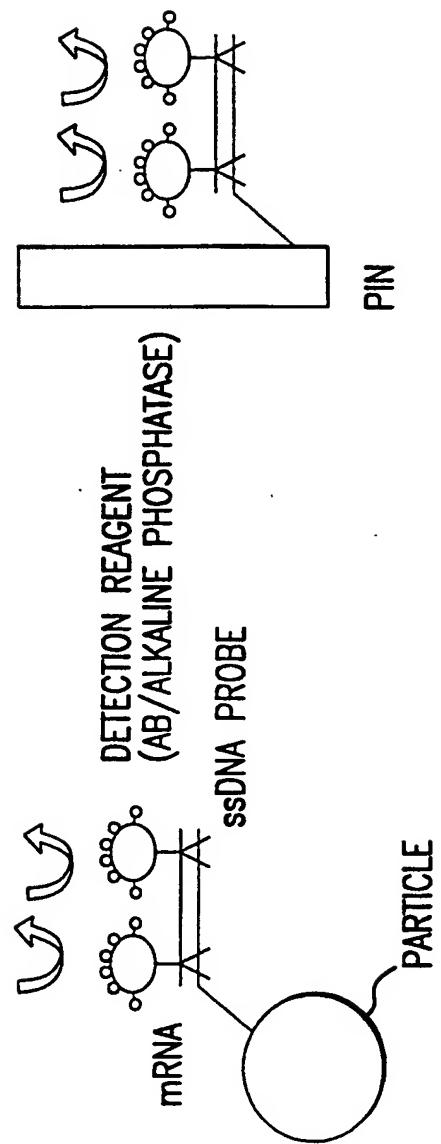


FIG. 1

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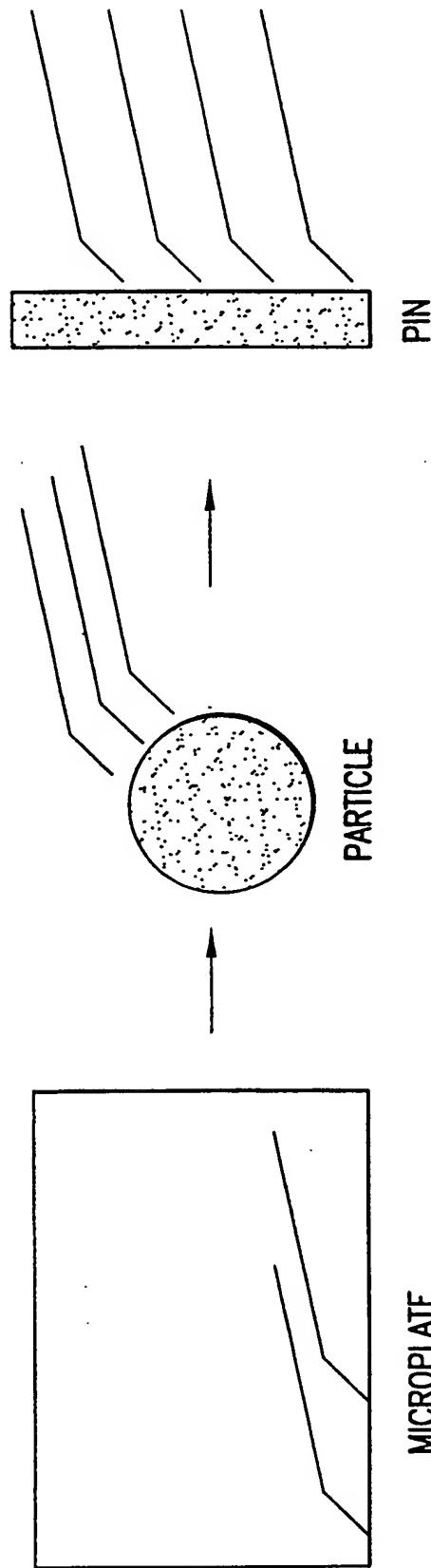


FIG. 2

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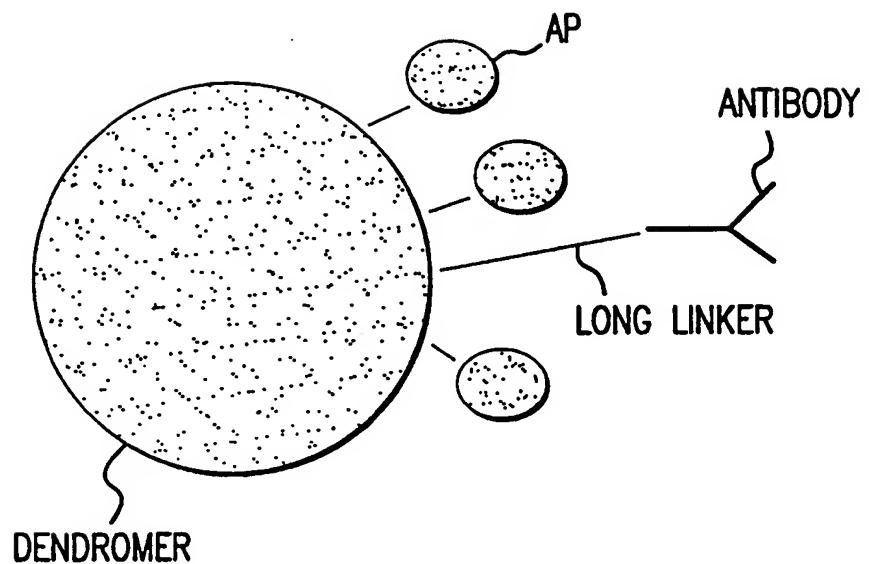


FIG.3

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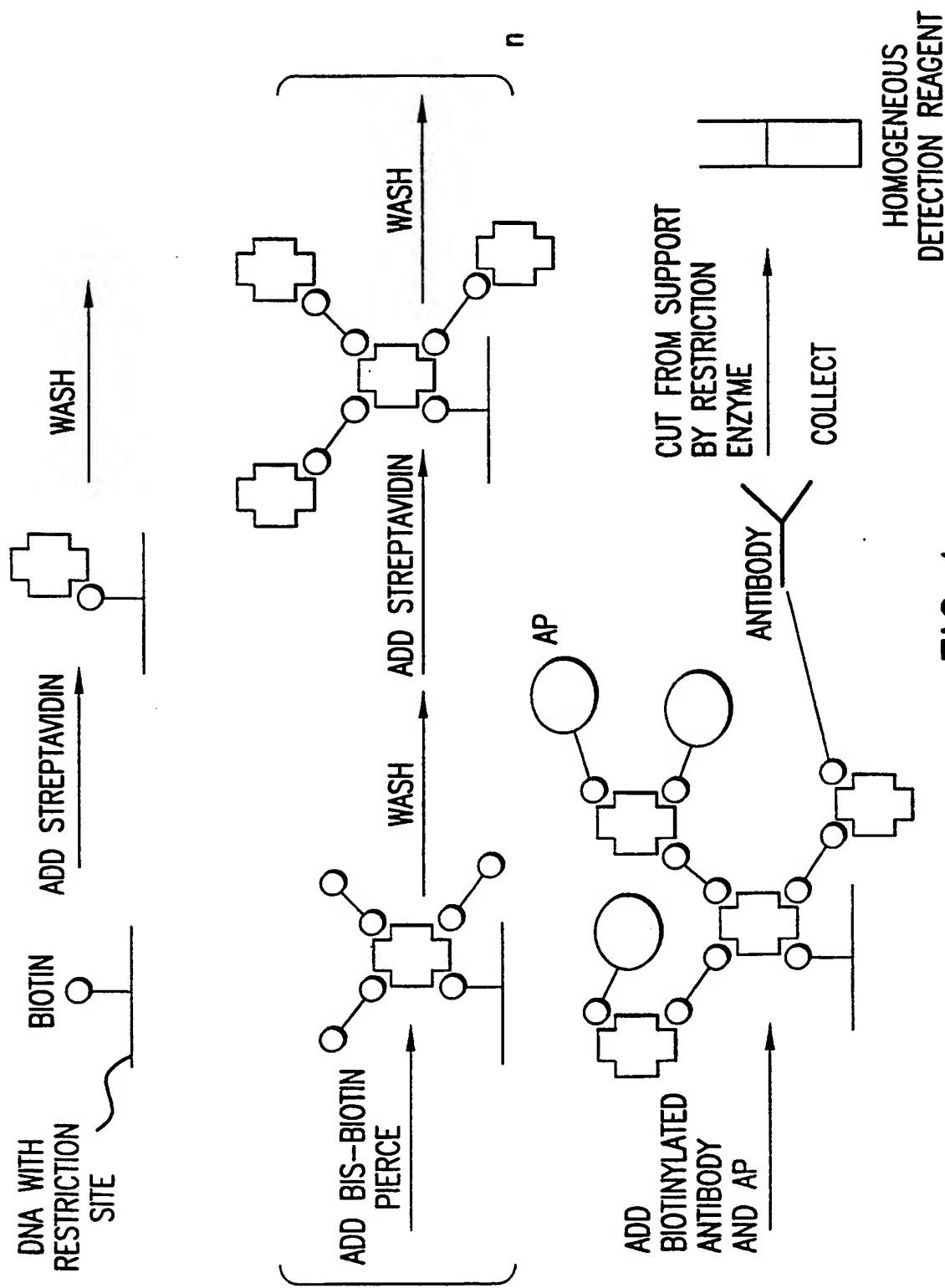
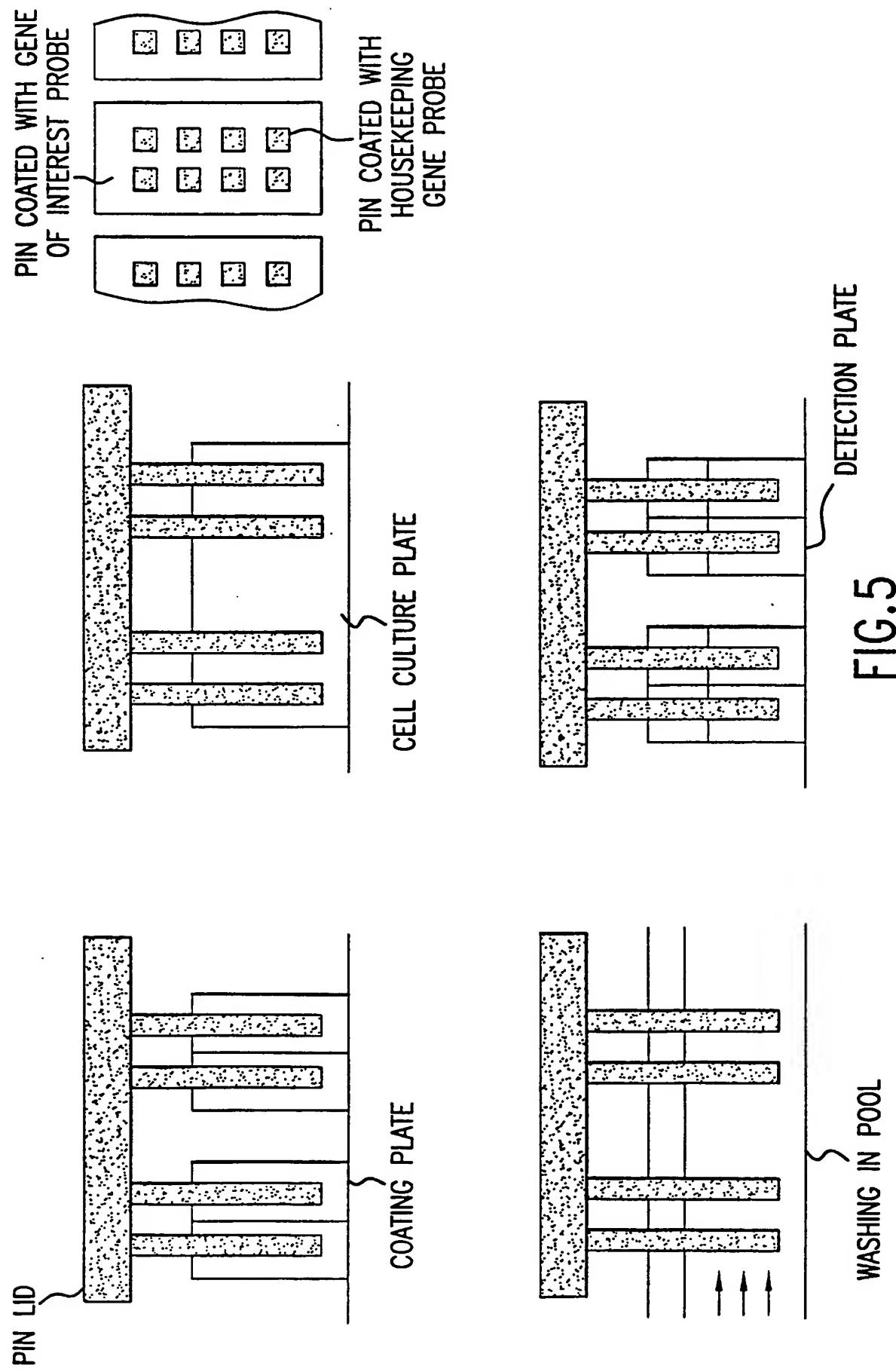
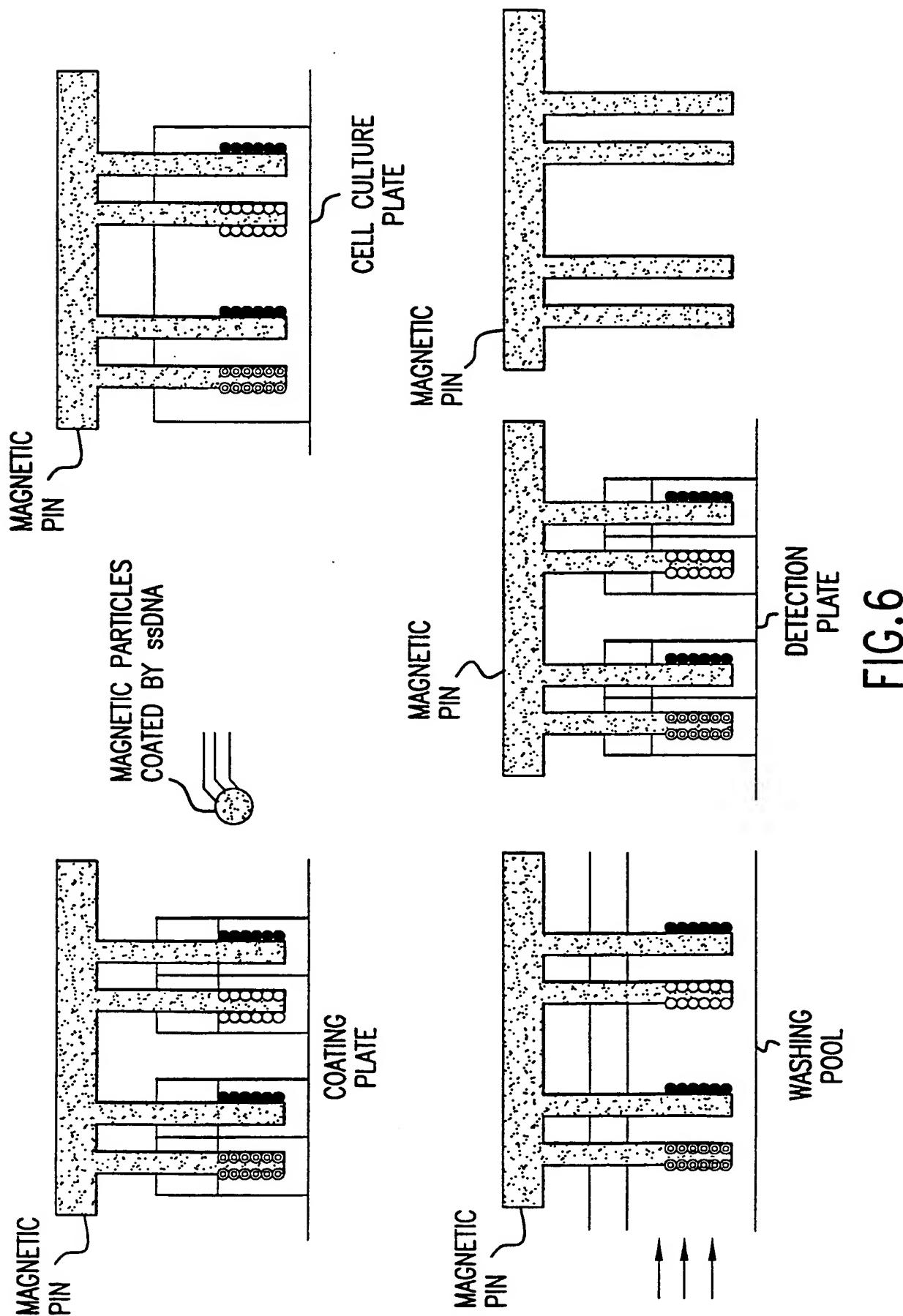


FIG.4

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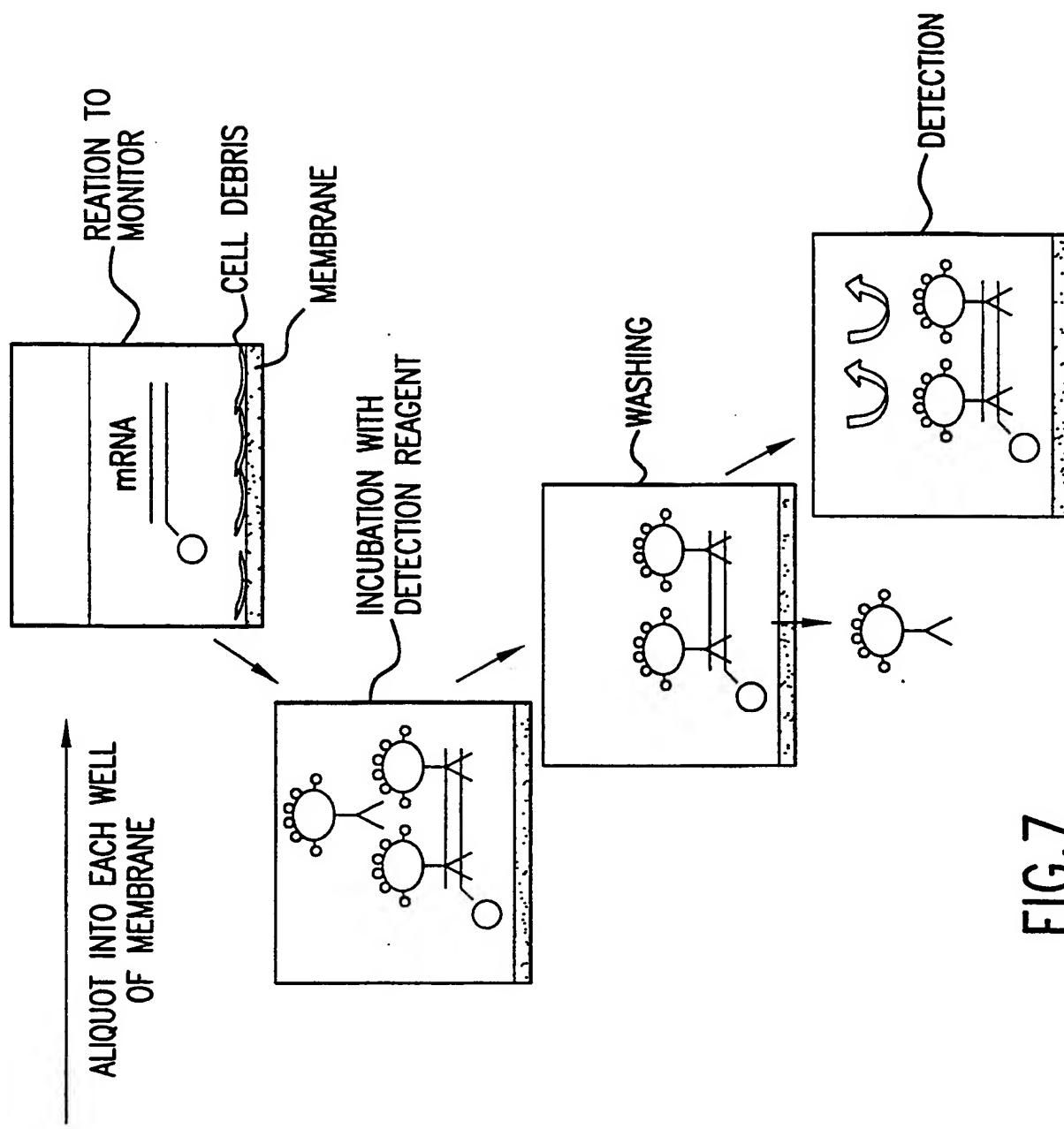
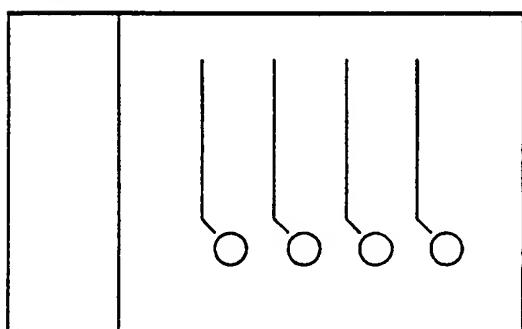


FIG. 7



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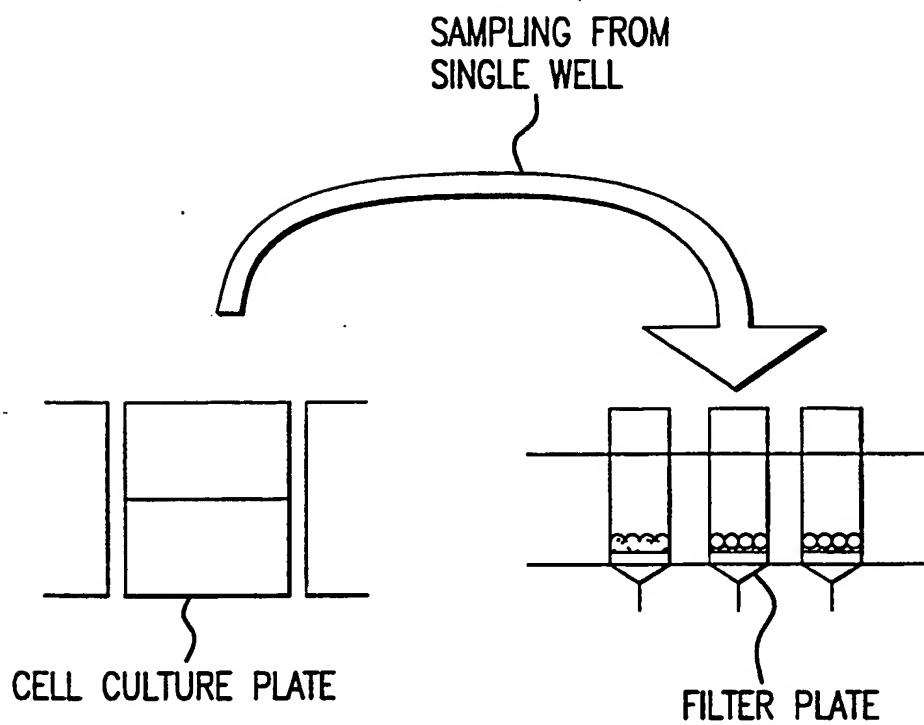


FIG.8

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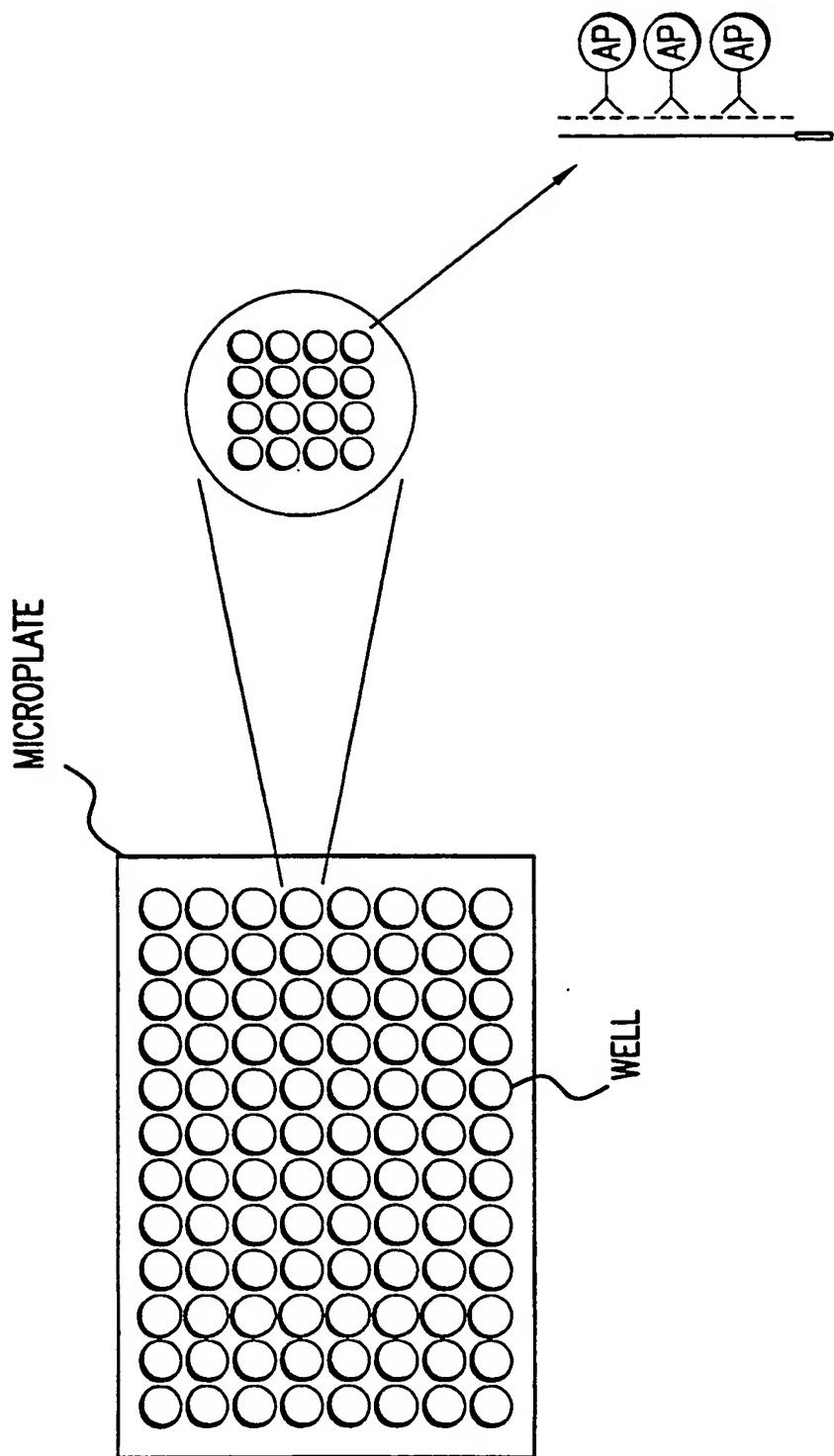


FIG. 9

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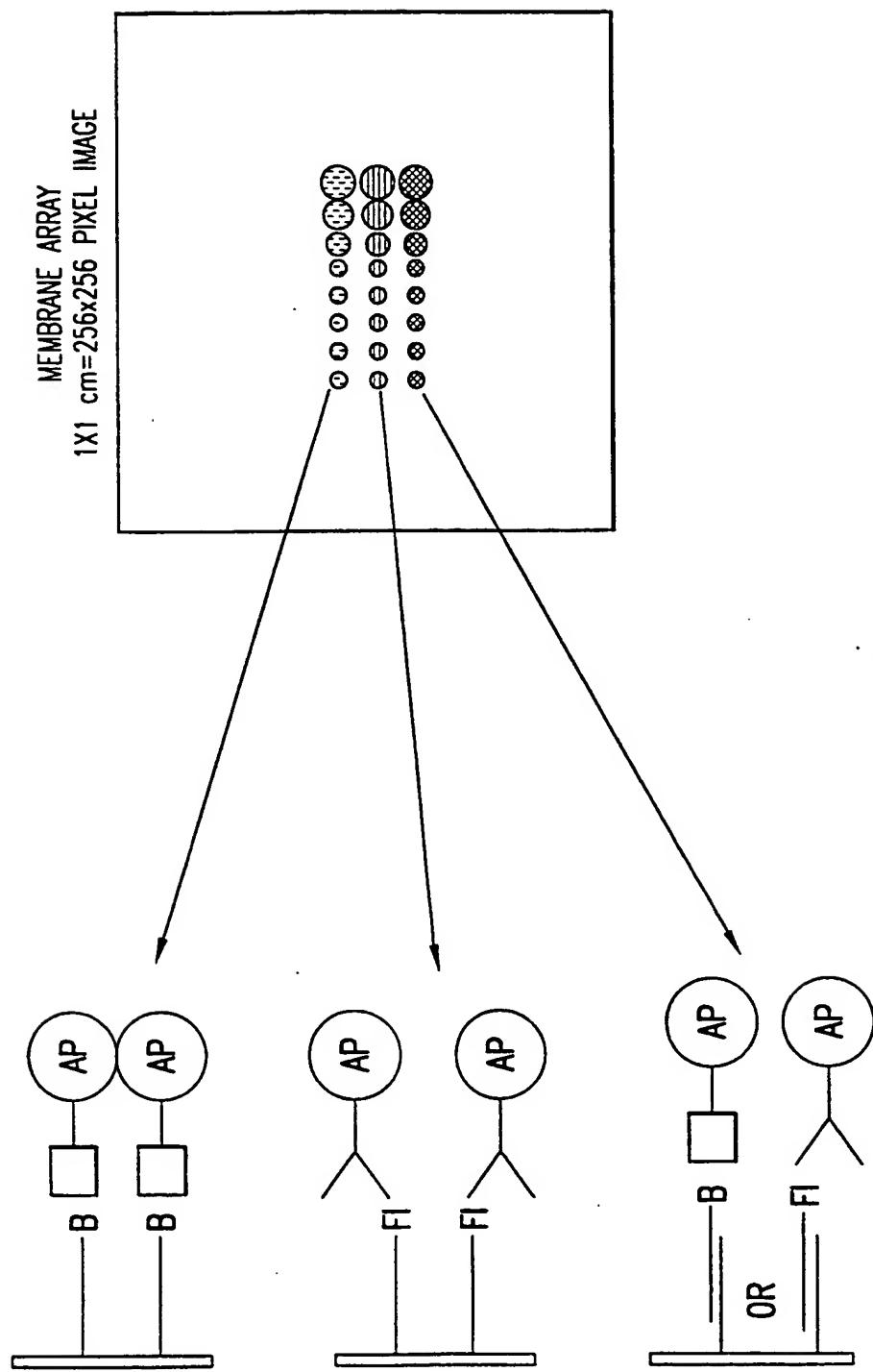


FIG. 10

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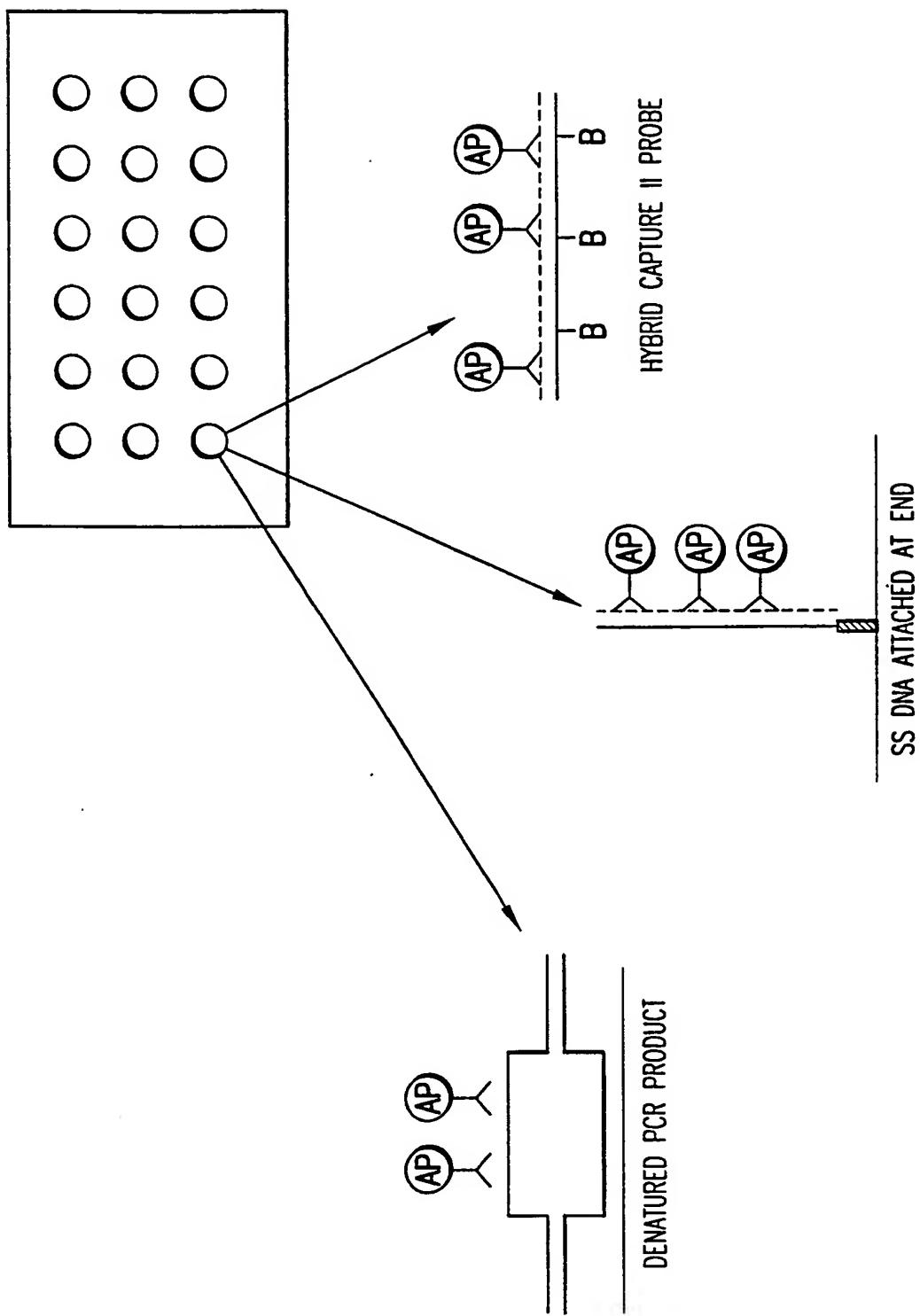


FIG.11

12/16

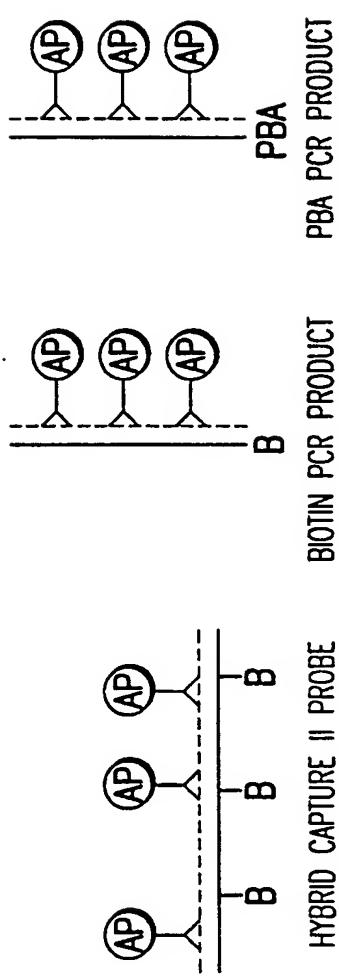


FIG. 12

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SOLUTION HYBRIDIZATION OF LONG PROBE
WITH CAPTURE AND DETECTION ON ZIP-CHIP

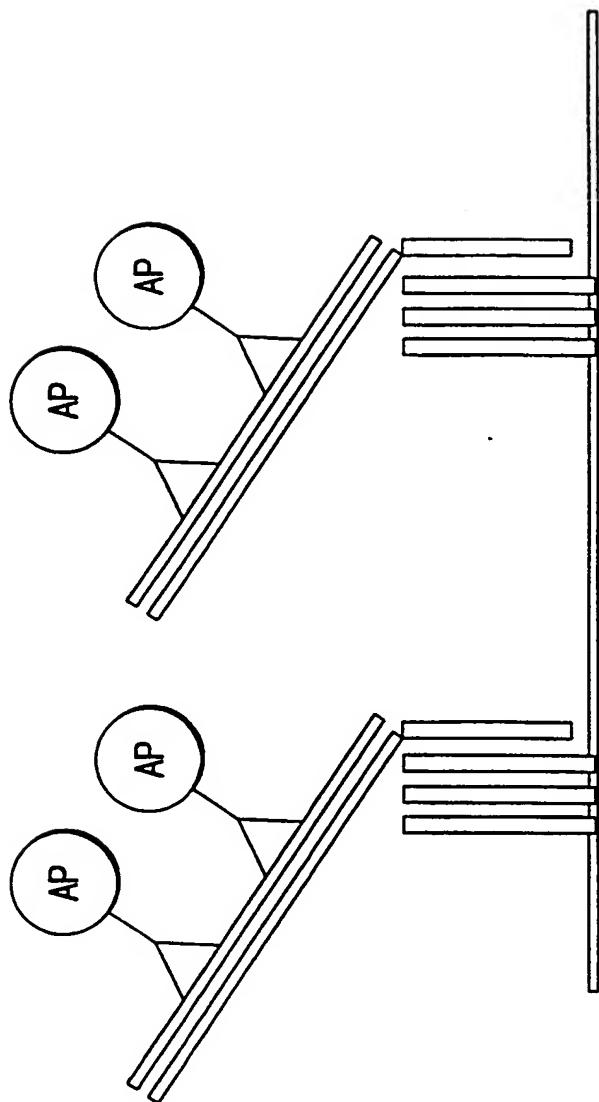
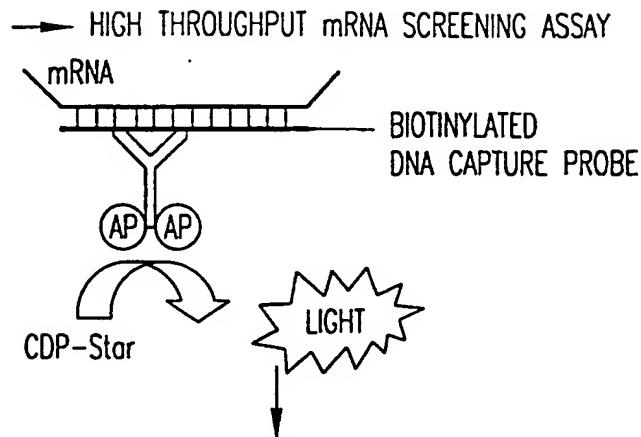


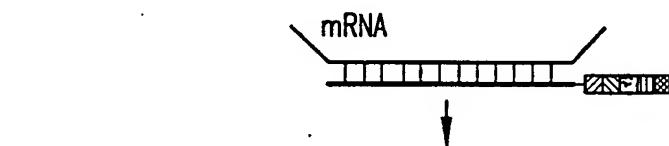
FIG. 13

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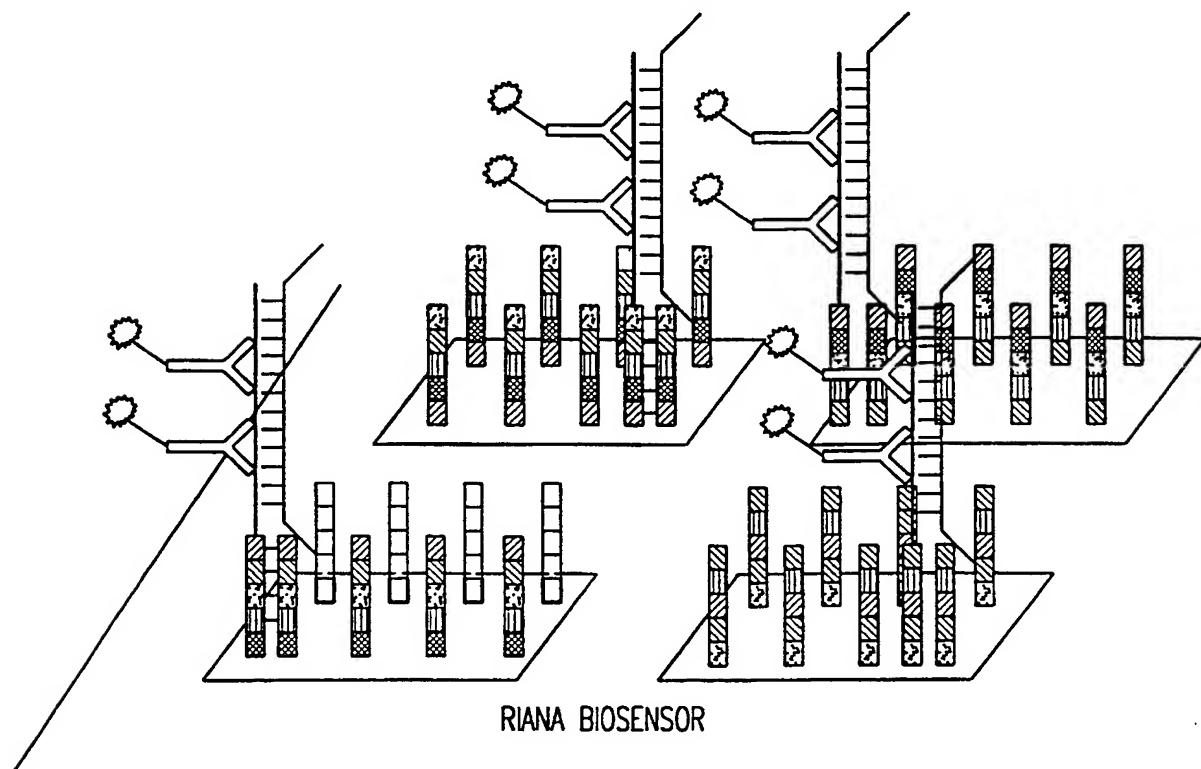


→ MODIFIED ASSAY USING THE RIANA-BIOSENSOR AS DETECTION DEVICE:

1. HYBRIDISATION OF mRNA AND FLUORESCENCE LABELED CAPTURE DNA TO FORM A DNA-RNA HYBRID



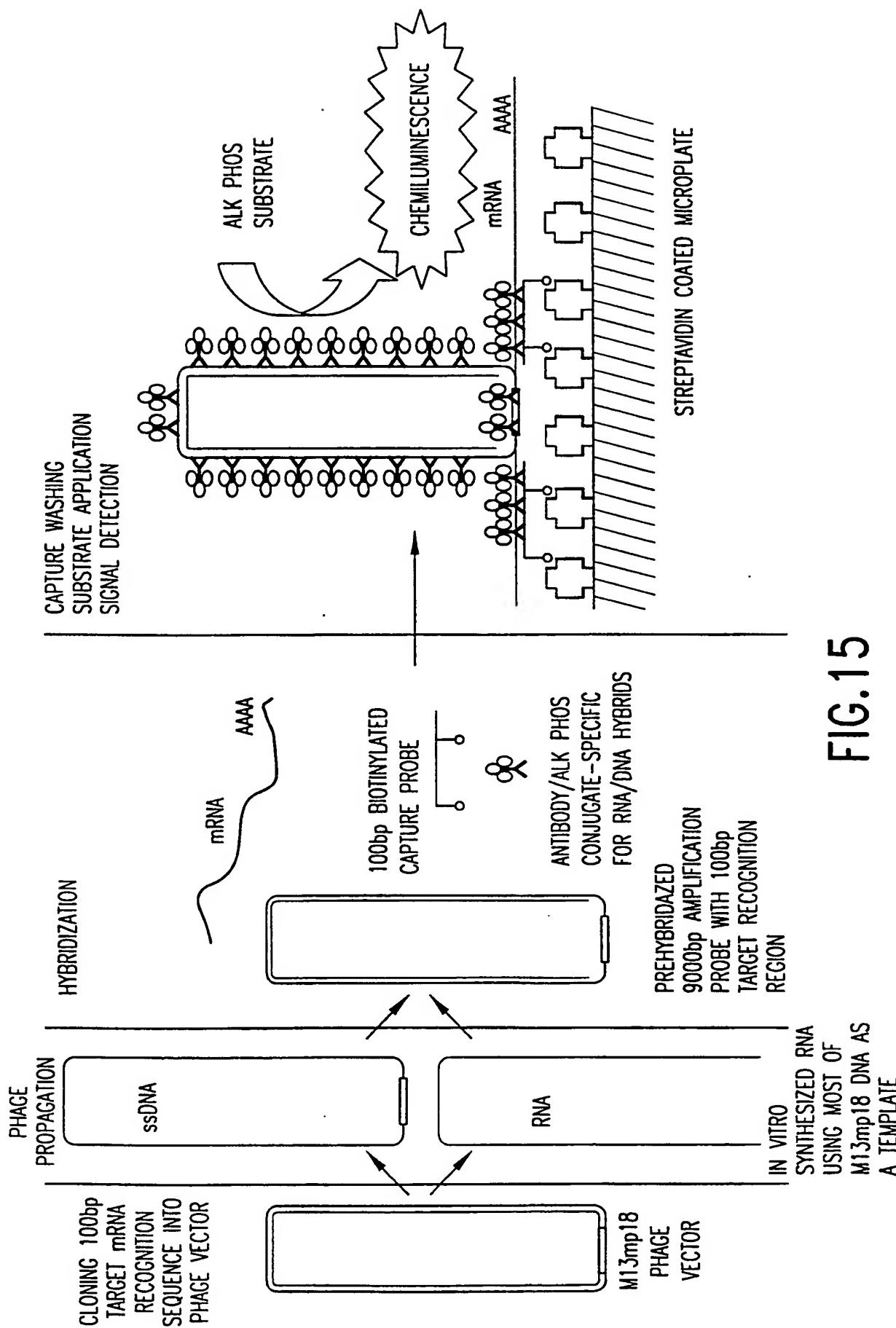
2. DECODING OF THE CAPTURE DNA-RNA HYBRID ONTO THE BIOSENSOR SURFACE USING THE ZIP-CODE



DETECTION BY USING FLUORESCENCE LABELED ANTIBODIES, HIGHLY SPECIFIC FOR RNA-DNA-HYBRIDS; MULTIPLEXING: 126 mRNA ARE DETECTABLE AT ONCE

FIG.14

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ARRAY FOR HIGH
THROUGHPUT
SCREENING

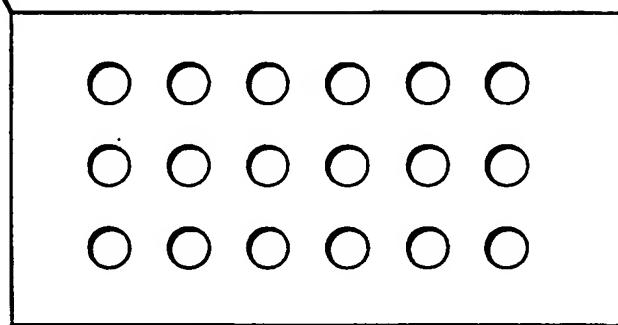


FIG.16

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/08465

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/68
US CL : 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6; 536/23.1, 24.5, 24.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP H1 178,933 A (HITACHI, LTD) 28 February 1991 (28.02.1995), see entire document	1-4, 7-10
X	US 5,006,473 A (BOUMA et al) 09 April 1991 (09.04.1991), especially column 3.	1-4, 7-18
X	KEENEY et al. "Mutation Typing using Electrophoresis and GelImmobilized Acrydite Probes" BioTechniques, September 1998, Vol. 25, No. 3, pages 516-521.	1-4, 7-18
X	US 5,770,367 A (SOUTHERN et al) 23 June 1998 (23.06.1998), see column 7-9.	1-7
X		
X, P	US 5,955,268 A (GRANADOS et al) 21 September 1999 (21.09.1999), see entire document	1-4, 7-18
X, P	US 5,976,797 A (MITSUHASHI) 02 November 1999 (02.11.1999), see entire document.	1-4, 7-20
X, P	US 6,027,886 A (LEYING et al) 22 February 2000 (22.02.2000), see entire document.	1-4, 7-18

Further documents are listed in the continuation of Box C. See patent family annex.

• Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 19 May 2000 (19.05.2000)	Date of mailing of the international search report 10 JUL 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Jeanine Enevold Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/08465

Continuation of B. FIELDS SEARCHED Item 3: WEST, MEDLINE, BIOSIS, CAPLUS, EMBASE
DNA, high throughput, array, microplate, mRNA, RNA, microtitre, drug, chemical, compound, express?